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(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office
Cardiff Road
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1. Your reference — AJB/JR/P32158

2. Patent application number
(The Patent Office will fill in this part) — **9822140.1** — 9 OCT 1998

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

SMITHKLINE BEECHAM PLC
NEW HORIZONS COURT, BRENTFORD,
MIDDLESEX TW8 9EP

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM

4. Title of the invention — Compounds

5. Name of your agent *(if you have one)*

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"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)*

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer yes if*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is named as an applicant, or
c) any named applicant is a corporate body.
See note (d)

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	5
Claim(s)	2
Abstract	
Drawings	

10. If you are also filing any of the following, state how many against each item.

Priority Documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 1/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. We request the grant of a patent on the basis of this application

Signature Alison Blakey Date 9-Oct-98

A J Blakey

12. Name and daytime telephone number of person to contact in the United Kingdom

A J Blakey 01279 644355

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Notes

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Compounds

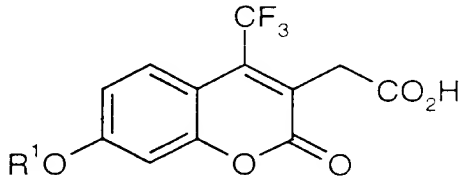
This invention relates to compounds, processes for preparing them and their use as enzyme substrates.

The majority of metabolism based drug interactions are a result of inhibition of cytochrome P450 enzymes. Drug interactions involving individual P450 enzymes can be predicted using *in vitro* methods. Typical *in vitro* P450 enzyme assays involve incubation of an appropriate substrate with a source of enzyme. Traditionally, time consuming chromatographic methods have been used for metabolite detection in these incubations. More recently the availability of fluorimetric plate readers has facilitated the higher throughput of enzyme assays in general. Adapting P450 assays to fluorescent plate reader technology requires the identification of substrates with appropriate fluorescent products for individual enzymes. Among the xenobiotic-metabolising cytochromes P450, CPY2C9 is one of those commonly responsible for the metabolism of drugs.

3-Cyano-7-ethoxycoumarin has been described for high throughput CYP2C9 inhibition screening (Crespi et al, Anal Biochem 1997; 248:188-190). However, the rate of 3-cyano-7-ethoxycoumarin metabolism by CYP2C9 is low and the extent of 3-cyano-7-ethoxycoumarin O-dealkylase inhibition does not always correlate well with a solid-phase extraction assay for CYP2C9, thus 3-cyano-7-ethoxycoumarin is not suitable for high throughput screening.

Certain compounds have now been identified which are improved substrates for CYP2C9 and which are of use for configuring high throughput inhibition screening assays.

According to the present invention there is provided an assay for testing for inhibitors of CYP2C9 which comprises contacting the enzyme and a compound of formula (I):



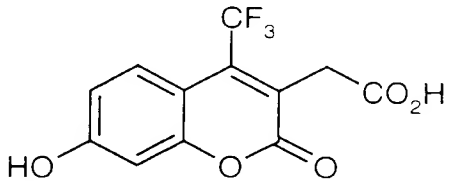
(I)

wherein R^1 represents C_{1-2}alkyl, with a test compound and measuring inhibition of O-dealkylation of the compound of formula (I) by the enzyme.

In a preferred aspect of the invention R^1 is methyl.

Generally the rate of O-dealkylation in the absence of test compound will be known, as will the extent of O-dealkylation at given time points. The assay may test for inhibition of O-dealkylation continuously or at specified time points.

O-Dealkylation of the compound of formula (I) following incubation with CYP2C9 gives a readily quantifiable fluorescent product of formula (II):



II

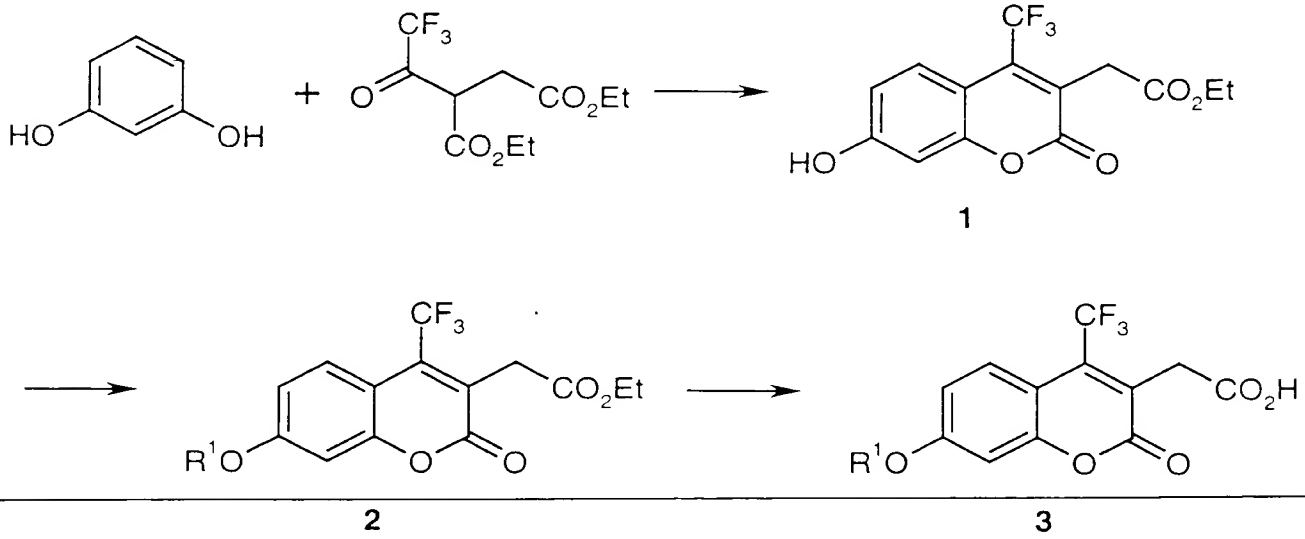
which can be scanned with suitable excitation and emission wavelengths, for example an excitation wavelength of 410 nm and an emission wavelength of 510 nm.

The assay may be carried out either in solution or utilising a solid support.

The test compound may be pre-incubated with enzyme prior to the addition of the substrate, or alternatively the substrate may be added simultaneously. Final concentrations of enzyme and substrate are calculated so as to achieve a suitable rate of processing for carrying out the assay. If desired, the reaction may be stopped, for example by addition of acid or solvent. The product may be analysed using any conventional system of fluorescence detection, for example a multi-well plate/fluorescent plate reader.

The compounds of formula (I) and (II) are novel and as such also forms part of the invention.

The compounds of formula (I) and (II) may be prepared by conventional methods, for example as shown in Scheme 1:



Scheme 1

Thus according to a further aspect of the invention there is provided a process for the production of a compound of formula (I) or (II) which comprises:

a) reaction of resorcinol and diethyl trifluoroacetosuccinate in the presence of polyphosphoric acid;

b) for compounds of formula (I) reaction of the resulting alcohol with a compound of formula R^1Hal, wherein Hal is halogen; and

c) ester hydrolysis to give the acid of formula (I) or (II).

The invention is illustrated by the following examples.

Example

Preparation of 7-methoxy-4-trifluoromethylcoumarin-3-acetic acid.

a) 7-Hydroxy-4-trifluoromethyl coumarin-3-acetic acid ethyl ester **1**

Polyphosphoric acid (45 g) was added to a mixture of resorcinol (4.06 g) and diethyl trifluoroacetosuccinate (9.96 g) (Aubert, C.; Begue, J. P.; Charpentier-Morize, M.; Nee, G.; Langlois, B. *J. Fluorine Chem.* **1989**, *44*, 361). The mixture was stirred at room temperature for 24 h. Crushed ice was added and the mixture extracted with dichloromethane. The organic phase was washed with water then dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica gel (eluent 2% methanol in dichloromethane) to give the title compound (1.24 g) m.p. 112.5 - 114.0°C. $\delta_H(CDCl_3)$ 1.30 (t, 3H), 3.96 (m, 2H), 4.24 (q, 2H), 6.66 (d, J = 2.5 Hz, 1H), 6.75 (dd, J = 9.0, 2.5 Hz, 1H), 7.55 (m, 1H); mass spectrum *m/z* 317 (MH^+).

b) 7-Methoxy-4-trifluoromethyl coumarin-3-acetic acid ethyl ester **2**

Methyl iodide (0.41 ml) was added to a mixture of **1** (0.7 g), potassium carbonate (0.46 g) and acetone (15 ml). The mixture was heated under reflux for 5 h. After cooling the solvent was evaporated and the residue was partitioned between dichloromethane and water. The organic phase was washed with water and then dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica gel (eluent 0.5% methanol in dichloromethane) to give the title compound (0.62 g). $\delta_H(CDCl_3)$ 1.26 (t, 3H), 3.90 (s, 3H), 3.94 (m, 2H), 4.19 (q, 2H), 6.86 (d, J = 2.6 Hz, 1H), 6.91 (dd, J = 9.2, 2.6 Hz, 1H), 7.70 (m, 1H); mass spectrum *m/z* 331 (MH^+).

c) 7-Methoxy-4-trifluoromethyl coumarin-3-acetic acid **3**

A mixture of **2** (0.615 g), ethanol (5 ml) and dilute hydrochloric acid (3M, 100 ml) was heated under reflux. After 6 h the mixture was cooled and the solid collected by filtration. The dried solid was crystallised from diethyl ether/hexane to give the title compound (0.475 g) m.p. 198.5 - 200.0°C (Found: C, 51.60; H, 2.82. $C_{13}H_9F_3O_5$ requires

C, 51.67; H, 3.00%); $\delta_H(CDCl_3)$ 3.90 (s, 3H), 3.94 (m, 2H), 6.87 (d, J = 2.6 Hz, 1H), 6.92 (dd J = 9.2, 2.6 Hz, 1H), 7.70 (m, 1H); mass spectrum *m/z* 325 ($M + Na^+$), 303 (MH^+), 285 ($M - OH^-$); m.p. 198.5 - 200.2°C.

Assay methodology

Materials:

6.25 mM 7-Methoxy-4-trifluoromethylcoumarin-3-acetic acid (i.e. 1.88 mg/mL in DMSO)
2 % (w/v) $NaHCO_3$ - store at approx. 4°C
50 mM potassium phosphate buffer, pH 7.4
Freshly prepared cofactor solution:- approx. the following per mL of 2 % (w/v) $NaHCO_3$

1.7 mg NADP, monosodium salt
7.8 mg glucose-6-phosphate, monosodium salt
6 Units glucose-6-phosphate dehydrogenase, Type VII from Bakers Yeast

1) Mix 1 uL 6.25 mM 7-methoxy-4-trifluoromethylcoumarin-3-acetic acid, 10 uL (100 ug) CYP2C9 microsomal protein and 209 uL buffer per incubate (giving 25 uM 7-methoxy-4-trifluoromethylcoumarin-3-acetic and 400 ug/mL protein final concentration).

2) To each well of a 96-well plate add 220 uL of incubation mix and 5 uL of compound in methanol.

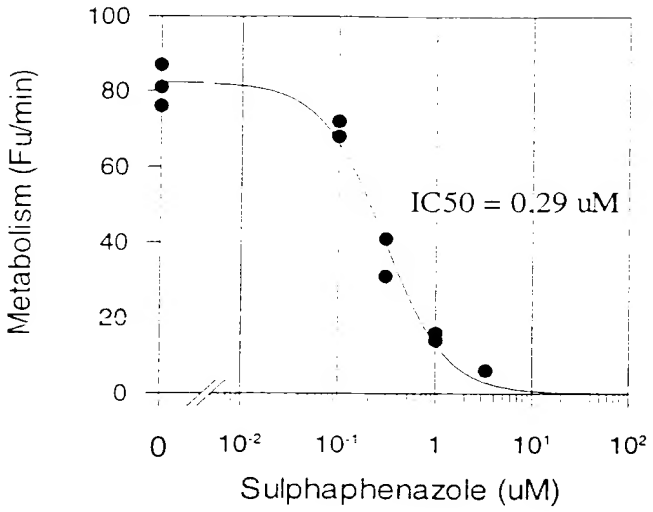
3) Pre-incubate the multi-well plate in the plate reader at 37°C for 5 minutes. Pre-warm the cofactor solution at 37°C for 5 minutes.

4) Add 25 uL cofactor solution to each well and scan with an excitation wavelength of 410 and an emission wavelength of 510 with a gain of 80. Scan for 10 cycles at 1 minute intervals.

Results

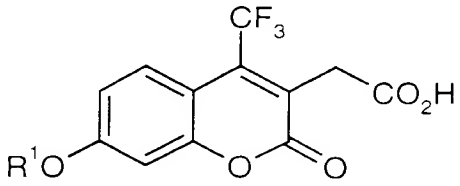
Confirmation of 7-methoxy-4-trifluoromethylcoumarin-3-acetic acid as a CYP2C9 substrate was achieved using sulphaphenazole, a diagnostic CYP2C9 inhibitor (Back *et al*, *British Journal of Clinical Pharmacology*, 1988, **26**, 23-29). With sulphaphenazole, 7-methoxy-4-trifluoromethylcoumarin-3-acetic acid was inhibited with an IC50 of 0.29 uM (Figure 1), an inhibition value typical of other, well characterised, CYP2C9 substrates.

Figure 1: Inhibition of 7-methoxy-4-trifluoromethylcoumarin-3-acetic acid



CLAIMS

1. An assay for testing for inhibitors of CYP2C9 which comprises contacting the enzyme and a compound of formula (I):

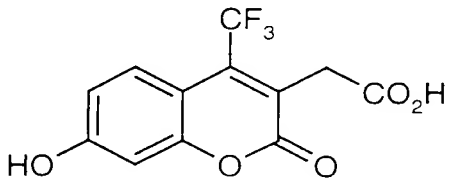


(I)

wherein R^1 represents C_{1-2}alkyl, with a test compound and measuring inhibition of O-dealkylation of the compound of formula (I) by the enzyme.

2. The assay according to claim 1 wherein R^1 is methyl.

3. The assay according to claim 1 or 2 wherein inhibition of O-dealkylation of the compound of formula (I) by the enzyme is measured by quantifying the compound of formula (II):



II

4. The assay according to claim 3 wherein the compound of formula (II) is quantified by fluorescence detection.

5. The assay according to claim 4 wherein the compound of formula (II) is quantified by scanning at excitation wavelength of 410 nm and an emission wavelength of 510 nm.

6. A compound of formula (I) or (II) as defined in claim 1 or 3.

7. A process for the production of a compound of formula (I) or (II) as defined in claim 1 or 3 which comprises:

a) reaction of resorcinol and diethyl trifluoroacetosuccinate in the presence of polyphosphoric acid;

b) for compounds of formula (I) reaction of the resulting alcohol with a compound of formula R^1Hal, wherein Hal is halogen; and

c) ester hydrolysis to give the acid of formula (I) or (II).